

Analysis of Mutagens from Cooked Foods by Directly Combined Liquid Chromatography–Mass Spectrometry

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Directly combined high performance liquid chromatography—mass spectrometry (LC/MS) has been studied as a method of analysis of heterocyclic aromatic mutagens in cooked foods, in the parts per billion concentration range. Identification and semiquantitative estimation of mutagens is based on accurate measurement of chromatographic retention (k') and molecular weight-selective detection of mutagens, which are protonated during passage of the chromatographic eluant into a thermospray interface of a quadrupole mass spectrometer. Standard chromatographic retention (k') values in two reversed-phase systems and data from thermospray mass spectra from nine mutagens are reported. An isolation scheme employing CH₃OH extraction, acid-base partition, cellulose–trisulfo-Cu-phthalocyanine adsorption, and normal-phase HPLC was used prior to LC/MS analysis. Initial applications have been demonstrated in the analysis of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) in broiled salmon flesh. Levels measured were estimated to be in the range 0.2 to 0.4 $\mu\text{g/kg}$ IQ and 0.4 to 0.9 $\mu\text{g/kg}$ MeIQ. The method is judged to be generally applicable with minimal sample prefractionation to detection of mutagens at the parts per billion level in cooked foods.

Introduction

The isolation and structural characterization of mutagens produced during the cooking of food (1) has constituted a significant step in identifying potential health hazards associated with dietary intake. Because of the trace levels at which these substances are formed, analytical methodology for their detection and quantitative estimation has assumed an unusually difficult, yet important, role. Nishimura has recently reviewed the methods used for analysis of mutagens in cooked foods (2). Mass spectrometry, often used in conjunction with off-line chromatography (3–6) or directly combined with gas chromatography (2), has been of particular importance because of its relatively high sensitivity, and its selectivity toward some structural features, such as mo-

lecular weight or elemental composition.

We report the results of an initial investigation of the use of directly combined high performance liquid chromatography–mass spectrometry (LC/MS) for analysis of the highly potent mutagens IQ and MeIQ (7,8). This approach is made possible by the development in recent years of the thermospray interface for LC/MS (9,10), which is particularly applicable to a range of polar compounds (11) and provides a major extension of the capabilities of either liquid chromatography or mass spectrometry alone. Detection is based both on precise measurement of chromatographic elution position, and on the mass or molecular weight of the mutagen in question. Because of the great selectivity afforded by the use of a mass spectrometer as a liquid chromatographic detector, interference levels for a mixture of given complexity are reduced compared with UV detection. As a consequence, normally laborious and time-consuming isolation procedures can be reduced. The results we describe indicate the general applicability of LC/MS for the analysis of heterocyclic amines in protein pyrolysates or other complex mixtures.

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Materials and Methods

High Performance Liquid Chromatography (HPLC)

Preparative and analytical HPLC was carried out using Beckman 332M liquid chromatographs, with UV (Waters 440 dual wavelength monitor for 254 and 280 nm) or mass spectrometric detection. Analytical separations were made using two reversed-phase systems: System 1: 4 × 300 mm μ Bondapak C-18, (Waters Associates) with 0.1 M ammonium formate, 0.01 M diethylamine, pH 4.5, and 40% CH₃CN-CH₃OH (4:1), 1.5 mL/min and System 2: 2 × 250 mm 5 μ Ultrasphere ODS (Beckman Instruments), with 0.1 M ammonium acetate, 0.02 M diethylamine, pH 5.6, and 30% CH₃CN-CH₃OH (4:1), 0.2 mL/min. For LC/MS experiments either a 4.6 × 250 mm 5 μ Ultrasphere ODS(IP) column or a 4 × 300 mm Waters μ Bondapak C-18 column was used, with 0.1 M ammonium formate, 0.01 M diethylamine, pH 4.5, 17.5% CH₃CN-CH₃OH (4:1), at 1.5 mL/min. directly introduced into the mass spectrometer. Preparative separations of IQ and MeIQ were made using System 3: normal phase 4 × 300 mm Waters μ Bondapak NH₂ column, with hexane-0.1% acetic acid in isopropanol (1:3), 1.5 mL/min.

Liquid Chromatography-Mass Spectrometry (LC/MS)

LC/MS experiments were carried out by use of a non-commercial quadrupole mass spectrometer with MAT 8 in. hyperbolic rods, a Vestec Corporation thermospray vaporizer probe and temperature controller, and a pumping configuration designed for LC/MS operation with thermospray (9,10) ionization. A Teknivent Corporation 29K Data System was used for instrument control and data acquisition and processing. Vapor temperatures of 310°C were used, for HPLC flow rates of 1.5 mL/min into the mass spectrometer. LC/MS measurements were made by selected ion monitoring of the protonated molecular ions for each mutagen: IQ, *m/z* 199; [²H₃]IQ, *m/z* 202; MeIQ, *m/z* 213; [²H₃]MeIQ, *m/z* 216.

Cooking of Meat and Partial Fractionation of Mutagens

The method of broiling meat and fish used in these studies closely approximates cooking procedures in a typical Japanese home. The food to be cooked is secured within a hinged grate, which can be hand-rotated in order to cook both sides. The grate is then placed over a broiling pan such that the surface of the meat or fish is approximately 2 cm from the bottom of the pan. The apparatus is then placed on a round gas-cooking burner adjusted to give a flame which just touches the surface of the pan.

Cooking times varied according to the thickness of the meat or fish. Salmon was sliced into 1.5-cm thick steaks for cooking. The inside of the fish was cooked, while the outside surfaces were well-browned, but not excessively charred. The cooked meat was ground and extracted three times with 500 mL of CH₃OH and the extract evaporated to dryness and weighed. The residue was dissolved in 250 mL 1 N HCl and extracted three times with 250 mL of CH₂Cl₂. The aqueous residue after extraction was adjusted to pH 10 and extracted three times with 250 mL CH₂Cl₂. The final organic extracts were combined, concentrated, and total mutagenicity measured using the *Salmonella typhimurium* strain TA 98 test. [²H₃]IQ and [²H₃]MeIQ internal chromatographic standards, previously synthesized (H. Kasai, unpublished experiments, 1982), were added at this point in quantities appropriate to levels estimated crudely from the Ames test or from previous experiments. When IQ and MeIQ levels are approximately known, the standards may be added to the initial crude CH₃OH extract. Starting with the 1 N HCl step, the acid-base partition was repeated, using solvent volumes reduced by 40%. The resulting residue was taken up in 70 mL H₂O and extracted using the method of Hayatsu (12) with 1-g portions of cellulose-trisulfo-Cu-phthalocyanine complex. The solid extracts were combined, washed with H₂O and extracted three times with 70 mL methanolic NH₄OH (50:1). A portion of the 1 to 2 mg residue was directly examined by LC/MS, or subjected to further normal phase purification by HPLC System 3.

Table 1. Liquid chromatographic, UV absorbance, and thermospray mass spectrometric data from mutagen reference compounds.

Compound	Molecular weight	<i>k'</i> ^a		UV absorbance ratio ^b <i>A</i> ₂₅₄ / <i>A</i> ₂₈₀	Thermospray mass spectrum MH ⁺ (% rel. int.)
		System 1	System 2		
2-Amino-3-methylimidazo[4,5- <i>f</i>]quinoline (IQ)	198	1.30	0.58	11.0	199 (100)
2-Amino-3,4-dimethylimidazo[4,5- <i>f</i>]quinoline (MeIQ)	212	1.37	0.88	6.19	213 (100)
2-Amino-3,8-dimethylimidazo[4,5- <i>f</i>]quinoxaline (MeIQx)	213	1.33	0.85	0.91	214 (100)
2-Amino-dipyrido[1,2- <i>a</i> :3'-2'- <i>d</i>]imidazole (Glu-P-2)	184	1.37	0.92	4.67	185 (100)
2-Amino-6-methyldipyrido[1,2- <i>a</i> :3'-2'- <i>d</i>]imidazole (Glu-P-1)	198	1.48	1.54	4.33	199 (100)
3-Amino-1-methyl-5H-pyrido[4,3- <i>b</i>]indole (Trp-P-2)	197	1.82	2.38	17.1	198 (100)
3-Amino-1,4-dimethyl-5H-pyrido[4,3- <i>b</i>]indole (Trp-P-1)	211	1.96	3.38	20.3	212 (100)
2-Amino-9H-pyrido[2,3- <i>b</i>]indole (AαC)	183	2.41	8.92	4.00	184 (100)
2-Amino-3-methyl-9H-pyrido[2,3- <i>b</i>]indole (MeAαC)	197	3.22	19.2	3.19	198 (100)

^a Solvent system 1, *t*_d = 1.06 min; solvent system 2, *t*_d = 2.6 min.

^b Solvent system 2, pH 5.6

Results and Discussion

High Performance Liquid Chromatography

Chromatographic retention properties in the form of k' values for nine heterocyclic mutagens are reported in Table 1. Chromatographic separations were studied using two analytically useful reversed-phase systems, thus providing greater latitude for exclusion of interferences and for increased confidence of identification with respect to k' values. In both Systems 1 and 2, chromatographic peak shapes for the heterocyclic bases were improved by addition of diethylamine (or triethylamine) as a competing base in the mobile phase. Relative concentrations of organic modifiers CH_3CN and CH_3OH were adjusted to optimize selectivity toward the compounds studied, for which a 4:1 ratio was found optimal.

The conditions chosen also maximize the separation of bases having very similar structures and therefore similar molecular weights, thereby complementing the use of a molecular weight-selective detector for HPLC

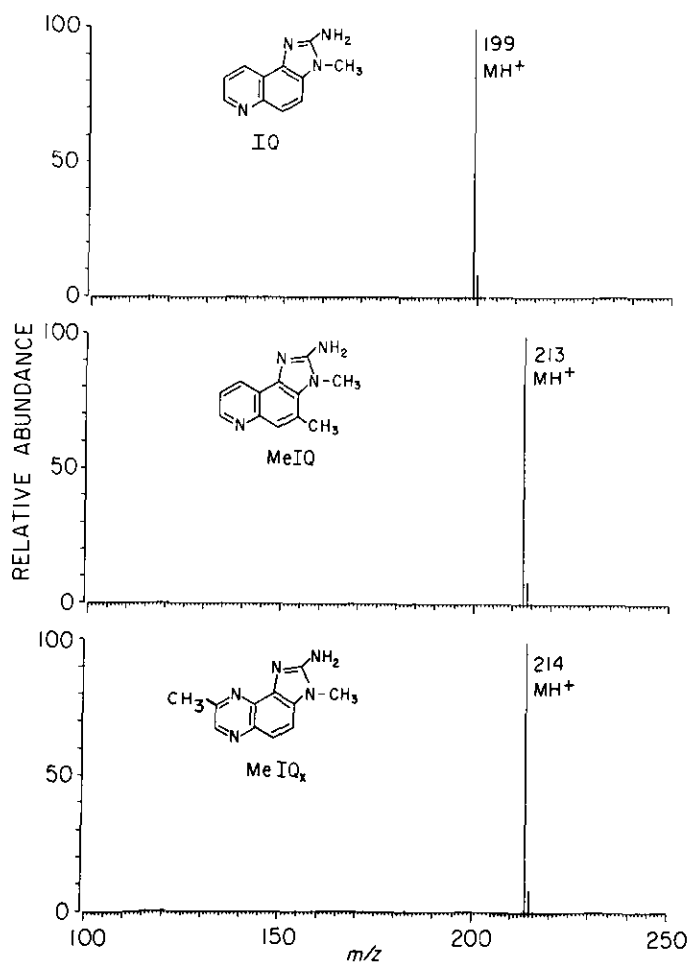


FIGURE 1. Thermospray mass spectra of IQ, MeIQ and MeIQx.

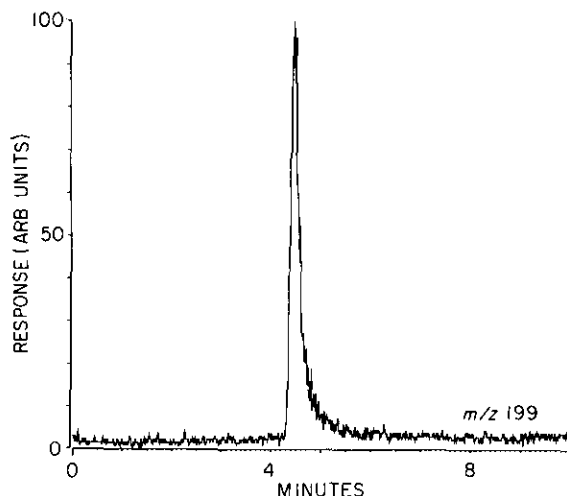


FIGURE 2. Selected ion recording from the MHb^+ ion from 1 ng of IQ, introduced by HPLC solvent System 2.

detection; e.g., MeIQ vs. MeIQx, IQ vs. Glu-P-1, or Trp-P-2 vs. MeAaC.

Ultraviolet absorbance ratios for 254 and 280 nm are highly characteristic for most of the compounds studied, as shown in Table 1. In specific instances more favorable wavelengths for monitoring can be used, depending on the mutagen involved, and the absorbance characteristics of interfering materials. In general, the use of A_{254}/A_{280} values for purpose of identification requires interference-free chromatographic profiles with adequate amounts of material for accurate ratio measurements, which in some cases may pose a limitation unless the mutagen has undergone extensive purification.

Thermospray Mass Spectrometry

Mass-abundance data for nine heterocyclic mutagens are given in Table 1, and full thermospray mass spectra

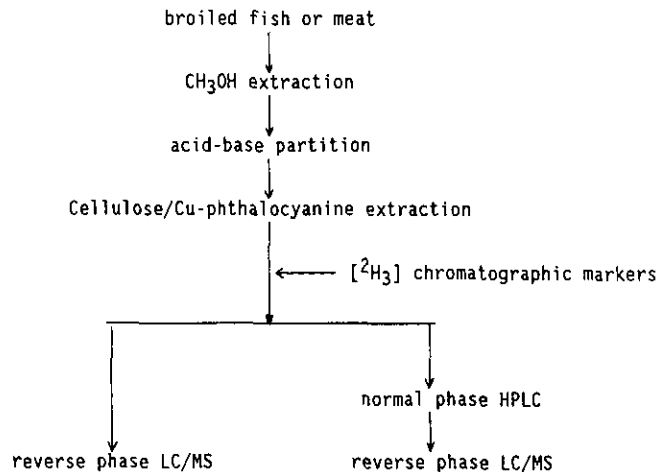


FIGURE 3. Scheme for extraction of mutagens for analysis by LC/MS.

of IQ, MeIQ, and MeIQx are shown in Figure 1. The thermospray process often produces abundant protonated molecular ions (9,10). As reflected in each case in Figure 1, the stability of the heteroaromatic nucleus and the basicity of the sample (proton affinity) leads to simple mass spectra in which the only peak is due to MH^+ .

As a consequence of these factors, and the fact that all ion current derived from each component is found in a single ion species, the sensitivity of detection of mutagens introduced by HPLC is high. Figure 2 shows the response from 1 ng of IQ, a typical result indicating good signal/noise ratio for subnanogram sample levels. However, in applications for trace analysis, such as the ppb-level concentrations of mutagens in cooked food, background interferences from extraneous material often present at levels orders of magnitude higher than the mutagens, become the limiting factor. In many conventional analyses by GC/MS or LC/MS with selected

ion monitoring, background interferences are often mitigated by monitoring several characteristic ions for each component. In the present case the simplicity of spectra produced precludes the latter approach, so that relatively greater reliance must be placed on chromatographic fractionation prior to LC/MS, and on the use of k' values to establish the exact elution position of the component of interest. In addition, as described in the following section, the elution positions can be accurately marked by monitoring 2H -labeled reference standards added prior to the last step.

Analysis of IQ and MeIQ in Salmon Flesh

The data reported in Table 1 and Figures 1 and 2 predict the suitability of LC/MS for the analysis of any of the mutagens listed in Table 1. IQ and MeIQ were chosen for initial applications for isolates from extracts of salmon flesh, and to determine the extent of possible

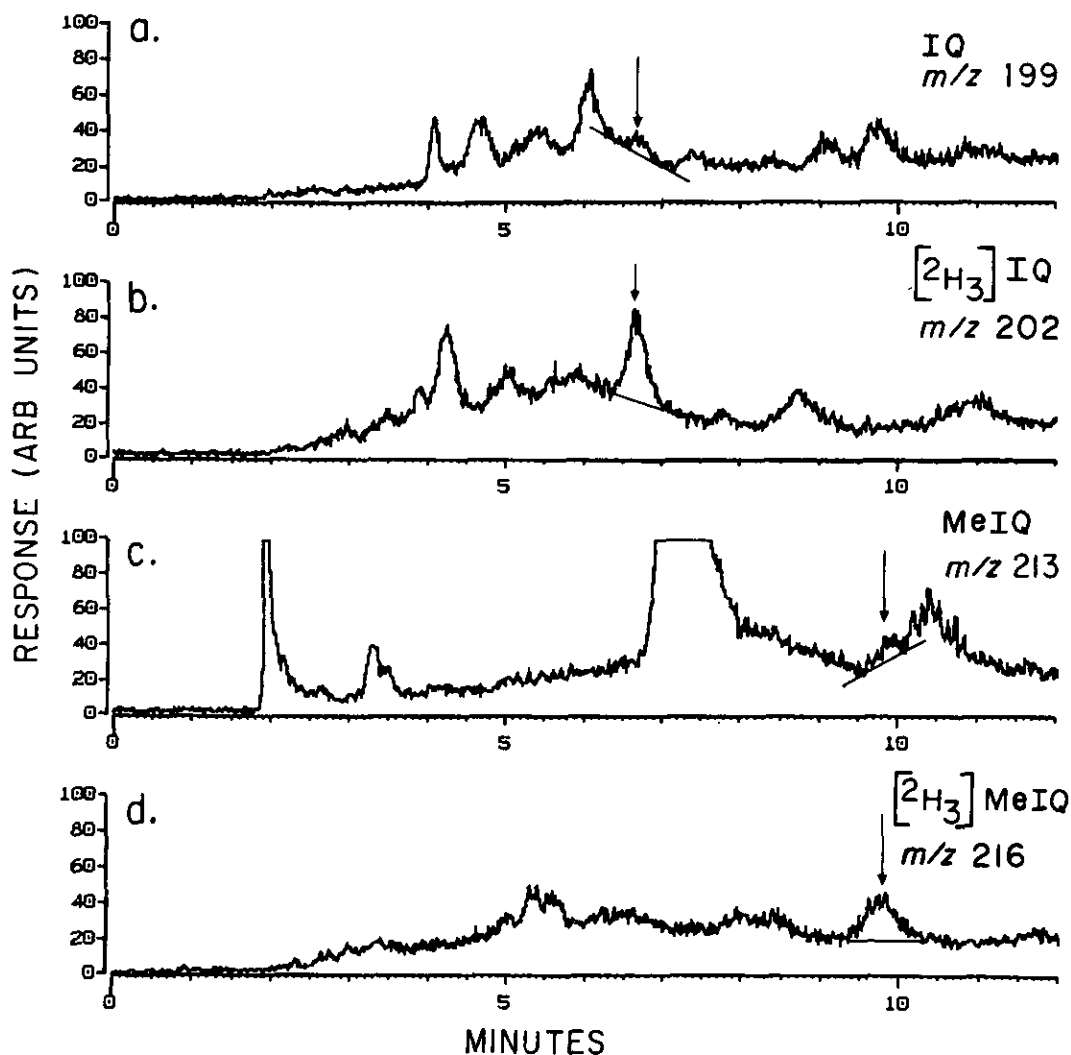


FIGURE 4. LC/MS analysis of IQ and MeIQ in extracts of broiled salmon flesh, without normal-phase HPLC prior to LC/MS. μ Bondapak C-18 column; conditions given in "Materials and Methods." Profiles shown were from MH^+ ions monitored during a single chromatographic run. Arrows show elution positions expected from k' values of reference compounds: (a) IQ; (b) $[^2H_3]IQ$; (c) MeIQ; (d) $[^2H_3]MeIQ$.

matrix interference for the isolation protocol used.

An isolation scheme was established, detailed in "Materials and Methods" and summarized in Figure 3, which provides sufficient concentration of IQ and MeIQ for detection by LC/MS, but is significantly shorter than procedures in which the component of interest is isolated in relatively pure form for characterization by mass spectrometry or other means. Following methanol extraction and acid-base partition, total mutagenicity in the preparation was analyzed by the Ames test in order to estimate the approximate amount of mutagens. The sample was then fractionated by the method of Hayatsu (12) which is highly selective for planar heterocycles such as IQ and MeIQ. Two alternate procedures were investigated, differing by use of a normal phase HPLC final clean-up step prior to LC/MS.

The addition of [methyl- $^2\text{H}_3$]IQ and [methyl- $^2\text{H}_3$]MeIQ as shown in Figure 3 provides chromatographic markers to establish unambiguously the elution positions of IQ and MeIQ by monitoring m/z values

three mass units higher than IQ and MeIQ.

Results from analysis of IQ and MeIQ from a single HPLC injection of a solution of 115 μg of residue, equivalent to 11.4 g of salmon flesh, are shown in Figure 4, from samples not subjected to normal phase HPLC as the final step. In each case the elution positions predicted by k' values (shown by arrows in Fig. 4) are verified by the selected ion recordings for the labeled markers, at m/z 202 for [$^2\text{H}_3$]IQ and m/z 216 for [$^2\text{H}_3$]MeIQ. The selectivity of mass spectrometric detection is demonstrated by the appearance of a large peak at approximately 7.3 min in Figure 4c. The corresponding peak is absent in the other selected ion recordings (Fig. 4 a, b, 4d) because the substance in question produces no ions at the mass values monitored in each of the other channels.

For comparison, results are shown in Figure 5 from a portion further purified using a normal phase NH_2 -column (right-hand branch in Fig. 3). A significant reduction in background in both the m/z 199 (IQ) and 213

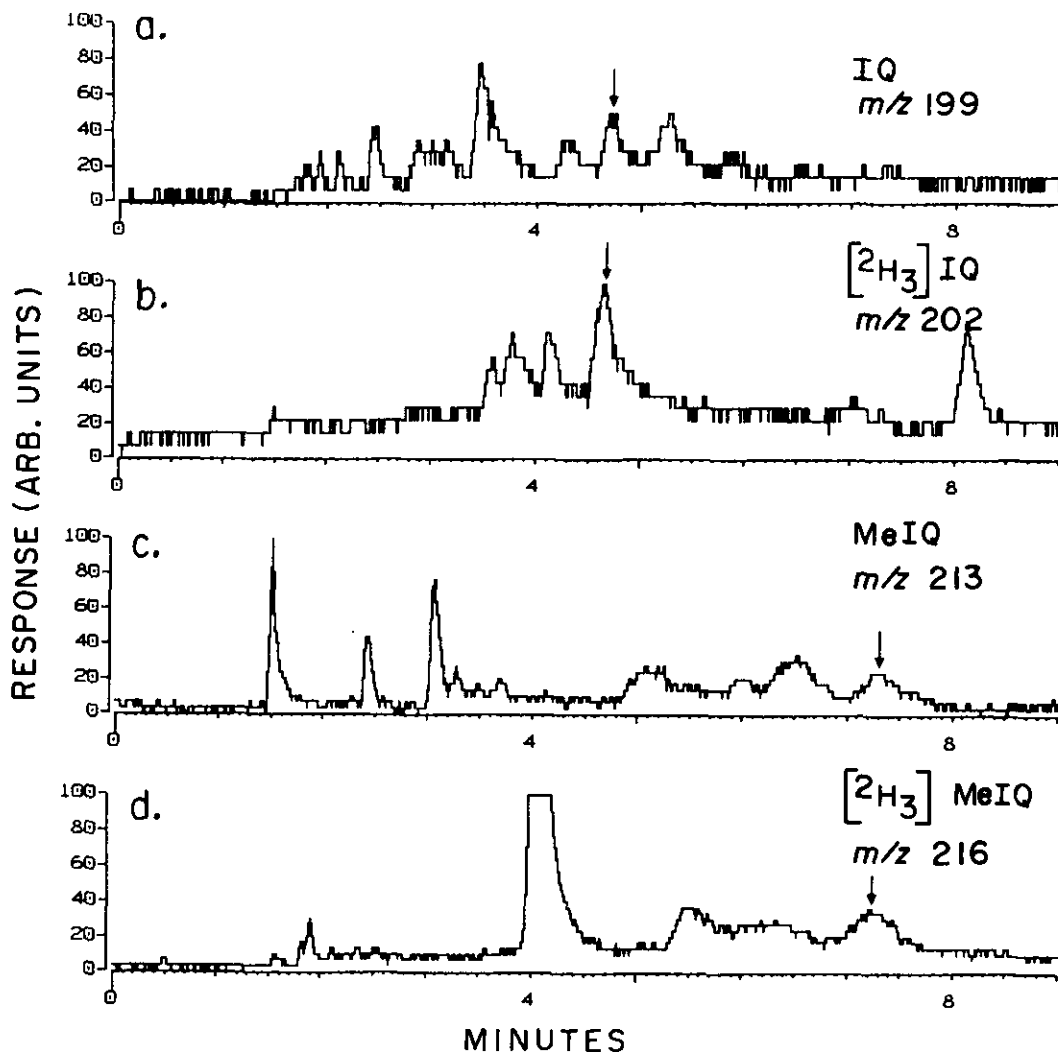


FIGURE 5. LC/MS analysis of IQ and MeIQ in the same sample represented in Figure 4, after normal-phase HPLC prior to LC/MS. Ultrasphere ODS-IP column; conditions given in "Materials and Methods."

(MeIQ) channels is seen in Figure 5. Based on responses from standard quantities of IQ and MeIQ obtained from separate LC/MS experiments, the quantities of IQ and MeIQ in Figures 4 and 5 are estimated to be in the range 0.5–1 ng IQ and 1–2 ng MeIQ, which are equivalent to 0.2–0.4 and 0.4–0.9 $\mu\text{g/kg}$ (ppb) of cooked salmon flesh, respectively.

These data demonstrate the great selectivity of detection which is provided by simultaneous measurement of chromatographic retention and mass, as well as the wide dynamic range that may be obtained. In practice, several tenths of a nanogram of IQ or MeIQ can be detected in a single analytical injection containing 200 μg of extract residue, corresponding to 0.001% of the injected material.

Further refinements in the method are anticipated by use of deuterated internal standards for quantitative estimation of mutagens. This approach requires addition of standards at an appropriate early stage of isolation, and demonstration through calibration curves that linear response can be obtained over the range of mutagen concentrations of interest. These studies are currently in progress in our laboratories (13).

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